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Award Number: W81XWH-08-1-0104

TITLE: The role of eIF4E activity in breast cancer

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REPORT DATE: August 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-2009	Annual	1 AUG 2008 - 31 JUL 2009

4. TITLE AND SUBTITLE
The role of eIF4E activity in breast cancer

5a. CONTRACT NUMBER
W81XWH-08-1-0104

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
University of Cambridge
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, MA 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Increased eIF4E expression occurs in many breast cancers and makes fundamental contributions to carcinogenesis by stimulating expression of cancer-related genes at post-transcriptional levels. This key role is highlighted by the facts that eIF4E levels can predict prognosis and that eIF4E is an established therapeutic target. However, eIF4E activity is a complex function of expression levels and phosphorylation statuses of eIF4E and its regulatory proteins. Our hypothesis was that combined analyses of these pathway components would allow insights into eIF4E activity and its influence on cancer.

We have established that mathematically combining assessments of expressions of eIF4E-regulators with assessments of expression of eIF4E in clinical tumours provides improved prognostic insights over examination of eIF4E alone. In doing so we have determined the mathematical relationships between expression of each pathway component and pathway activity. Using human cell lines, we have experimentally demonstrated that this combinatorial estimate of eIF4E activity does indeed reflect eIF4E activity in breast cells. This represents a critical validation of our technique for estimation of eIF4E activity and allows us to propose that the estimate may act as a potentially powerful therapy predictive marker for cancer therapies directed against the eIF4E pathway.

5. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER

1. Introduction

This project is a synergistic award. The biological investigations are taking place at the university of Leeds supervised by Dr. Hughes. Dr. McElwaine is supervising the mathematical analysis at the university of Cambridge.

Increased expression of eIF4E has frequently been reported in breast cancers and is thought to make fundamental contributions to disease development and progression[1]. Increased eIF4E activity acts to enhance the translation of oncogenic cancer-related transcripts that contain highly structured 5' untranslated regions (UTRs) in their mRNAs. Over-expression of eIF4E has been shown to correlate with poor prognosis in breast cancer[2] therefore the level of eIF4E over-expression has been studied extensively as a prognostic marker with some success. Furthermore, eIF4E is an established target for cancer therapy[3] and clinical trials of the efficacy and safety of cancer therapeutics that target eIF4E have been carried out, again, with some success. However, it is clear that eIF4E expression may not equate to eIF4E activity, since eIF4E activity is additionally regulated by a family of binding proteins, the 4E-BPs, that bind to and inhibit eIF4E activity[4].

Hypothesis*: analysis of eIF4E* ***activity*** *in individual breast tumours, as opposed to eIF4E expression, will give improved prognostic, predictive and biological understanding of individual breast cancers, and overall insights into the mechanisms of breast carcinogenesis.*

In order to test this hypothesis we aimed to investigate eIF4E activity in breast carcinogenesis using a novel cross-disciplinary approach. First, we aimed to examine the expression levels of eIF4E and the 4E-BPs, and the activity of eIF4E in cultured human cells using various biological assays. Secondly, we aimed to determine a mathematical relationship between these expression levels and eIF4E activity. Finally, we aimed to determine expression levels of eIF4E and its regulators in breast tumours, and to estimate eIF4E activity using the mathematical analyses above. Our expectation was that this measure of activity would provide a more effective prognostic marker than using eIF4E expression alone. In addition, we expected that the measure of eIF4E activity would provide an effective predictive marker for appropriate targeting of therapies directed against eIF4E. We have made substantial progress on each of these aims.

2. Body

1) Determination of expression levels of eIF4E and its regulators in cell lines (section 4.2a of narrative/section A3 of Statement of Work).

We have determined relative expression levels of eIF4E, 4E-BP1, 4E-BP2 and phospho-4E-BP1 (Thr37/46) in a panel of human cell lines (HB2, MCF7, MDA-MB-231, MCF-10A, A549, H1299, U2020, Caco-2, SW480) using Western blots with two alternative cell lysis methods (RIPA lysis, normalized to total protein; Laemmli lysis normalized to expression of beta-actin) (Fig 1). Assessment of expression in these lines using immuno-histochemistry is underway (4E-BP1, 4E-BP2 and phospho-4E-BP1 are complete; data for eIF4E will be completed shortly). Interestingly, we find that assessments by the different techniques give quite different answers. Using the mathematical analyses in section 4 we have been able to establish which is most representative of the levels of the 'active' proteins.

2) Determination of eIF4E activity in cell lines (narrative 4.2b/SoW A4 and A5).

We have determined relative eIF4E activity in the panel of cell lines (as above) both using a methyl-7-GTP-Sepharose cap-binding assay (data not shown), and by determining translation efficiency of reporter mRNAs with a variety of structured 5'UTRs[5-7] (Fig 2). As in section 1, we find that these assessments with different techniques give quite different answers, but – again - using the mathematical analyses we have been able to establish which is most representative of actual eIF4E activity.

3) Examination of archival breast tumours (narrative 4.2c/SoW A1).

We have determined expression levels of eIF4E, 4E-BP1, 4E-BP2 and phospho-4E-BP1 (Thr37/46) in a cohort of breast tumours supported by extensive clinical background and follow up. We have established the relationship between expression of these markers and tumour grade, size and type[8]. Moreover, we have combined the insights gained from each of these markers into an estimate of eIF4E activity (see below).

4) Mathematical analyses (narrative 4.2d/SoW B1 and B3).

We have developed a mathematical function that relates expression levels of eIF4E, 4E-BP1, 4E-BP2 and phospho-4E-BP1 in clinical breast tumours to survival. This function, '*z*', provides additional prognostic insights when compared to examination of eIF4E expression

levels alone[8]. This variable can be described as X–B1/4+PB1/2-B2/4, where X, B1, PB1 and B2 represent eIF4E, 4E-BP1, phospho-4E-BP1 and 4E-BP2 levels respectively. Using data from section 1 above, we have determined relative levels of *z* in cell lines for each of the detection methods used. We have compared these with the various measures of eIF4E activity from section 2 above. By determining which measure of *z*, and which experimentally determined measure of eIF4E activity correlate most closely we have established the methodologies that are most representative of the levels of the 'active' proteins (RIPA extractions, normalized to total protein) and actual eIF4E activity (a reporter assay for translational efficiency of mRNAs containing a 60 nucleotide hair-pin loop within the 5'UTR; "+60", see[5]; note that this reporter is the only one used here which contains a single well-characterized structural motif and no upstream ORFs). As this correlation is surprisingly close (Fig 3), especially in breast cell lines, we have been able to validate *z* as a true estimate of eIF4E activity in breast cells.

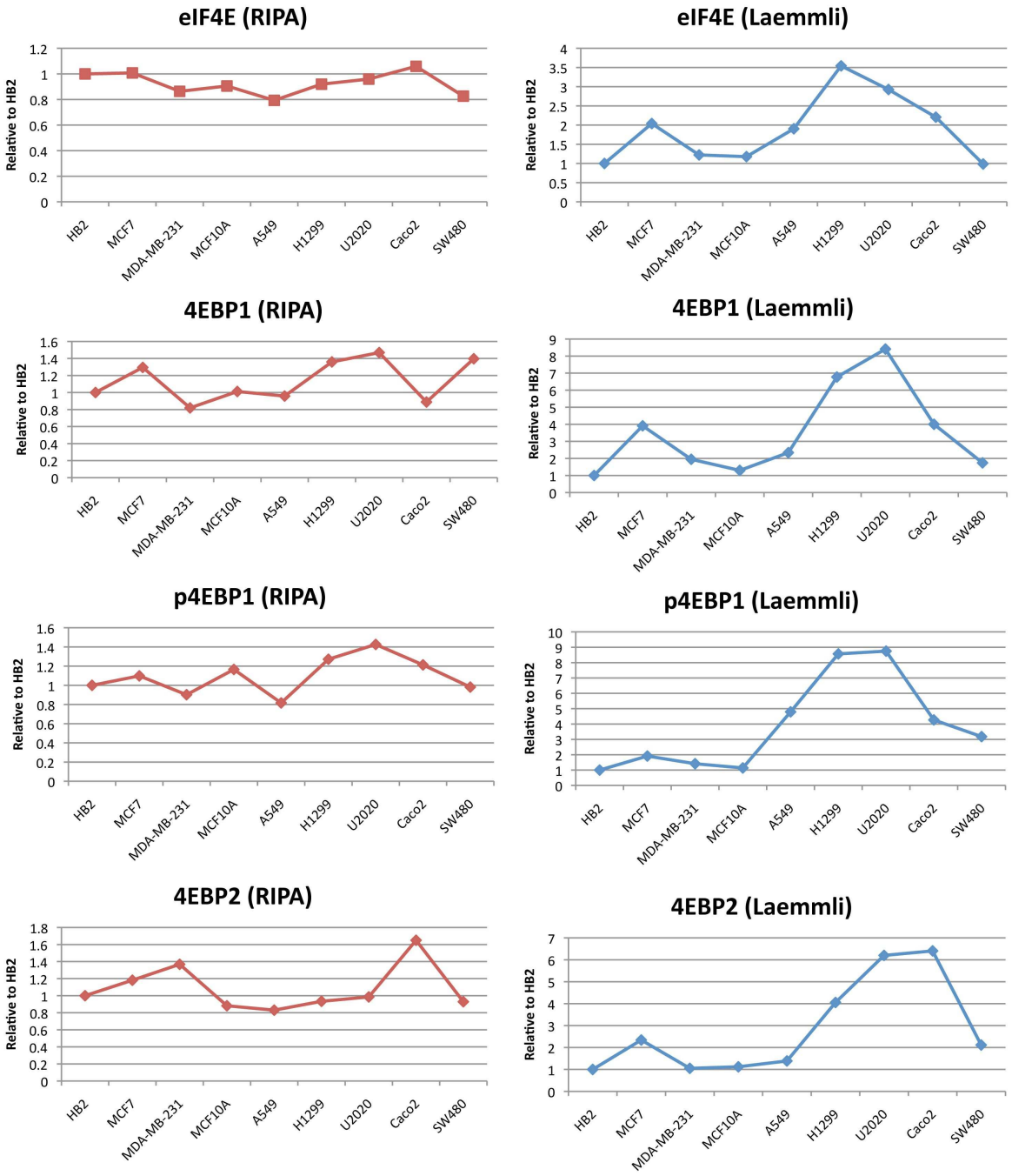

Figure 1 Relative expressions of eIF4E, 4E-BP1, phospho-4E-BP1 and 4E-BP2 were determined by densitometric analyses of Western blots using either RIPA extracts normalized to total protein (RIPA) or Laemmli extracts normalized to expression of beta-actin (Laemmli).

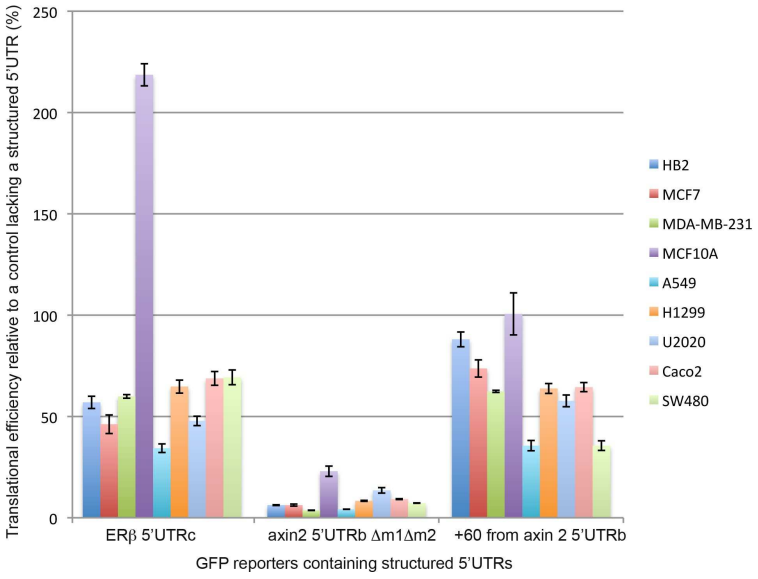

Figure 2 Relative translational efficiencies for reporter mRNAs with different structured 5'UTRs as shown. See[7] for an explanation of the determination of relative translational efficiencies and[5, 7] for a description of these specific reporters.

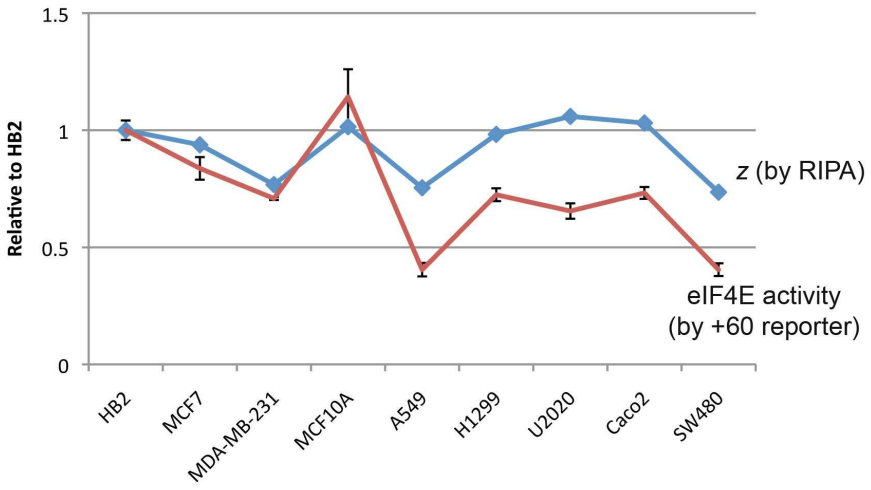

Figure 3 eIF4E activity was determined experimentally (red line; see Figure 2 +60 reporter) or estimated using the *z* variable and relative expressions of the pathway components as measured by Western blot using RIPA extracts (blue line; see Figure 1).3**. Key Research Accomplishments**

1) We have established that mathematically combining assessments of expressions of eIF4E-regulators with assessments of expression of eIF4E in clinical tumours provides improved prognostic insights over examination of eIF4E alone, presumably as this combinatorial measure reflects eIF4E *activity*. This work has recently been published[8].

2) Using cell lines, we have experimentally demonstrated that this combinatorial estimate of eIF4E activity does indeed reflect eIF4E activity. This represents a critical validation of our technique for estimation of eIF4E activity and allows us to propose that it may act as a potentially powerful therapy predictive marker for therapies directed against the eIF4E pathway, such as sirolimus[9], everolimus[10] and 4E-ASO[11].

4. Reportable outcomes

After the first year of funding, this work has led directly to one publication:

Coleman LJ, Peter MB, Teall TJ, Brannan RA, Hanby AM, Honarpisheh H, Shaaban AM, Smith L, Speirs V, Verghese ET, McElwaine JN & **Hughes TA** (2009) Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity. Br J Cancer 100, 1393-9

5. Conclusions

We have already tested and supported our main hypothesis: analysis of eIF4E *activity* in breast tumours does give improved prognostic insights into breast cancer over analysis of only eIF4E expression.

This conclusion and our methodology have some useful implications for breast cancer treatment.

1) As eIF4E *activity* correlates with poor prognosis it is reasonable to assume that eIF4E contributes to this poor prognosis. This is different from many correlative observations of expression where it is unclear as to whether the molecule in question is functionally related to cancer behavior or merely correlates with it. This supports the use of eIF4E as a target for cancer therapy.
2) Our estimate of eIF4E activity may provide a potentially powerful therapy predictive marker for therapies directed against the eIF4E pathway, such as sirolimus[9], everolimus [10]and 4E-ASO[11].

In the forthcoming year of funding we will continue to examine eIF4E regulation in the context of breast cancer, and aim to determine the potential therapy predictive value of our estimate of eIF4E activity.

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